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THE OPTIMIZATION OF THE ENZYME IMMOBILIZATION METHODS FOR AMPEROMETRIC GLUCOSE BIOSENSORS

(PENGOPTIMUMAN KAEDAH PENYEKAT-GERAKAN ENZIM UNTUK PENDERIA GLUKOSA AMPEROMETRIK)

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ABSTRACT

Enzyme immobilization method affects the performance of a glucose biosensor considerably. In this project, several methods of glucose oxidase immobilization for peroxide based amperometric glucose biosensor had been investigated. Parameters such as temperature, matrix concentration and cross-linker concentration were considered. For the first enzyme immobilization method, the effect of casting temperature on apparent enzyme activity was investigated. Glucose oxidase was immobilized in cross-linked poly(vinyl alcohol) at two different temperatures, 25^{o}C and 4^{o}C. The membranes immobilized at 25°C showed higher enzyme activity after 25 days compared to membranes immobilized at 4°C. The membranes immobilized at 25°C were also found to be able to retain enzyme better. For the second enzyme immobilization method, the effect of matrix concentration on apparent enzyme activity was investigated. Glucose oxidase was immobilized in cross-linked poly(vinyl alcohol) at two different PVA concentrations, 10% PVA and 15% PVA. With higher PVA concentration (15%), the enzyme retaining ability was better. However, the 10% PVA-GOD membranes performed better in terms of available enzyme activity. Enzyme activity of the 10% PVA-GOD membranes was approximately 33% higher than the 15% PVA-GOD membranes. For the third enzyme immobilization method, the effect of matrix concentration on apparent enzyme activity was once again investigated. However, this time physical enzyme immobilization method was considered. Glucose oxidase was immobilized in freeze-thawed PVA at three different PVA concentrations, 5% PVA, 10% PVA and 15% PVA. This work suggests that the higher the PVA concentration used for immobilization, the better the retention of the enzyme. Nevertheless, higher PVA

concentration didn't necessarily correlate well with enzyme activity. 10% freeze-thawed PVA-GOD membranes have the highest activities. The performances of 15% freeze-thawed PVA-GOD membranes and 5% freeze-thawed PVA-GOD was comparable. In terms of kinetic properties, PVA-GOD freeze-thawed membranes prepared with 10% PVA exhibited the highest K_m^{app} compared to the others. This means that they are more suitable to be used as bio-recognition elements for glucose biosensors than the other two. For the fourth enzyme immobilization method, the effect of cross-linker concentration on apparent enzyme activity was investigated. Glucose oxidase was immobilized within silica sol/PVA and cross-linked with (3-glycidoxypropyldimethylethoxy)silane. For the membranes prepared with 1:1 (TMOS: 3GPDES), the percentage of enzyme activity which remained at day 40 was about 51%. Meanwhile, for the membrane prepared with 1:2 (TMOS: 3GPDES) and 1:3 (TMOS: 3GPDES), the percentage of enzyme activity which remained at day 40 was 69% and 58%, respectively. V_{max}^{app} and K_m^{app} values for membranes prepared with 1:2 (TMOS: 3GPDES) were the highest indicating that it is most suitable to be used as the bio-recognition element for a glucose biosensor due to its stability and kinetic properties.

ABSTRAK

Kaedah penyekat-gerakan enzim boleh memberikan kesan yang agak besar terhadap prestasi biosensor glukosa. Dalam projek ini, beberapa kaedah penyekat-gerakan enzim glukosa oksides bagi biosensor glukosa amperometrik yang berdasarkan peroksida telah dikaji. Beberapa parameter seperti suhu, kepekatan matriks dan kepekatan penyambung-silang telah diambil kira. Bagi kaedah penyekat-gerakan enzim yang pertama, kesan suhu tuangan terhadap aktiviti nyata enzim telah dikaji. Glukosa oksides telah disekat-gerak dalam poli(vinil alkohol) disambung-silang pada dua suhu yang berbeza iaitu 25^{o}C dan 4^{o}C. Membran yang disekat-gerak pada 25^{o}C menunjukkan aktiviti enzim yang lebih tinggi selepas 25 hari berbanding membran yang disekat-gerak pada 4^{o}C. Membran yang disekat-gerak pada 25^{o}C juga mampu menyekat-gerak enzim dengan lebih baik. Bagi kaedah penyekat-gerakan enzim yang kedua, kesan kepekatan matriks terhadap aktiviti nyata enzim telah dikaji. Glukosa oksides telah disekat-gerak dalam poli(vinil alkohol) disambung-silang pada dua kepekatan PVA yang berbeza iaitu 10% PVA dan 15% PVA. Kepekatan PVA yang lebih tinggi (15%) mampu menyekat-gerak enzim dengan lebih baik. Namun begitu, membran PVA-GOD 10% mempunyai prestasi yang lebih baik dari segi aktiviti enzim yang sedia ada. Aktiviti enzim membran PVA-GOD 10% adalah lebih kurang 33% lebih tinggi dari membran PVA-GOD 15%. Bagi kaedah penyekat-gerakan enzim yang ketiga, kesan kepekatan matriks terhadap aktiviti nyata enzim telah dikaji sekali lagi. Namun begitu, kali ini kaedah penyekat-gerakan enzim yang digunakan ialah kaedah fizikal. Glukosa oksides telah disekat-gerak dalam PVA dibeku-cair pada tiga kepekatan PVA yang berbeza iaitu 5% PVA, 10% PVA dan 15% PVA. Kerja ini mengesyorkan bahawa kepekatan PVA yang lebih tinggi yang digunakan untuk proses penyekat-gerakan akan mampu menyekat-gerak enzim dengan lebih baik. Namun, kepekatan PVA yang lebih tinggi

tidak semestinya mempunyai korelasi yang baik dengan aktiviti enzim. Membran PVA-GOD dibeku-cair 10% mempunyai aktiviti yang paling tinggi. Prestasi membran PVA-GOD dibeku-cair 15% dan membran PVA-GOD dibeku-cair 5% adalah hampir sama. Dari segi sifat kinetik, membran PVA-GOD dibeku-cair yang dihasilkan menggunakan 10% PVA menunjukkan K_m^{app} yang paling tinggi berbanding yang lain. Ini bermakna membran ini lebih sesuai digunakan sebagai elemen pengenalpasti unsur biologi untuk biosensor glukosa berbanding dua kaedah sebelum ini. Bagi kaedah penyekat-gerakan enzim yang ke empat, kesan kepekatan penyambung-silang terhadap aktiviti nyata enzim telah dikaji. Glukosa oksides disekat-gerak dalam silika sol/PVA dan disambung-silang dengan (3-glaisidoksipropildimetiletoksi)silan. Bagi membran yang disediakan menggunakan 1:1 (TMOS: 3GPDES), peratusan enzim aktiviti yang tinggal selepas hari ke 40 ialah lebih kurang 51%. Bagi membran yang disediakan menggunakan 1:2 (TMOS: 3GPDES) dan 1:3 (TMOS: 3GPDES), peratusan enzim aktiviti yang tinggal selepas hari ke 40 ialah 69% dan 58%. V_{max}^{app} dan K_m^{app} bagi membran yang disediakan menggunakan 1:2 (TMOS: 3GPDES) adalah yang paling tinggi menunjukkan bahawa membran ini paling sesuai digunakan sebagai elemen pengenalpasti unsur biologi untuk biosensor glukosa kerana kestabilannya dan sifat kinetiknya.

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3GPDES	-	(3-glycidoxypropyldimethylethoxy) silane
A	-	Ampere
BCA	-	Bicinchoninic Acid
BSA	-	Bovine Serum Albumin
CGM	-	Continuous Glucose Monitoring
DCCT	-	Diabetes Control and Complications Trial
FAD	-	Flavin Adenine Dinucleotide
FDA	-	U.S. Food and Drug Administration
FHD	-	Flavin Hypoxanthine Dinucleotide
GOD	-	Glucose Oxidase
H_2O	-	Water
HCI	-	Hydrochloric Acid
K_2HPO	-	Potassium Hydrogen Phosphate
KH_2PO_4	-	Potassium Dihydrogen Phosphate
K_m	-	Michaelis Menten Constant
K_m^{app}	-	apparent Michaelis Menten Constant
NaOH	-	Natrium Hydroxide
PVA	-	Poly(vinyl alcohol)
TMOS	-	Tetramethoxysilane
U	-	unit
UKPDS	-	United Kingdom Prospective Diabetes Study
V	-	voltan
V_m	-	maximum velocity
YSI	-	Yellow Springs Instrument Company

CHAPTER 1

INTRODUCTION

1.0 Introduction

Diabetes is a disease in which the body loses the ability to regulate the level of glucose in the blood. In a person without diabetes, the body is able to regulate the amount of glucose in the blood between 3.5 to 6.5 mM with the help of insulin. In diabetes, the auto-regulation of glucose fails and the blood glucose level of a diabetic sufferer may vary between 1 to 30 mM . This can lead to hyperglycemia (too much glucose) or hypoglycemia (too little glucose). Hyperglycemia can result in long term damage to organs and hypoglycemia can result in coma or death due to too little glucose reaching the brain.

Diabetics have to monitor their blood glucose closely in order to remain healthy and to decrease the risks of serious complications of the disease. The Diabetes Control and Complications Trial (DCCT), which began in 1983 and stretched over a period of 10 years, examined the effect of tight regulation of glucose levels on the complications suffered by type 1 diabetics [1]. The study followed two groups of over 1441 diabetes sufferers. One group used a standard regime of glucose

measurements and insulin shots and the other group maintained tight control over their blood glucose level by checking their blood glucose levels and injecting themselves with insulin more frequently. Over time, the group that monitored their blood glucose levels more closely suffered less complications related to the disease compared to the other group. A computer simulation that was performed based on the DCCT results predicted a longer life span and a better quality of life for diabetics who controlled their blood glucose level tightly. The computer program estimated that patients who regulate their blood glucose level tightly can expect an additional 5 years of life, 6 years free of amputations, 8 years of sight, and 6 years free from kidney disease [2]. Another study that was carried out on non-obese type 2 Japanese diabetic patients showed that intensive insulin therapy prevented the advancement of microvascular complications, which supported the results of the DCCT [3]. Recently the results of a new study, the United Kingdom Prospective Diabetes Study (UKPDS) were released. As in the two previous studies, this study demonstrated similar effects of an intensive insulin treatment policy on type 2 diabetic patients [4].

The studies on intensive insulin treatment demonstrated that the health of diabetics depends on their ability to tightly control their blood glucose levels. Fewer health complications will decrease the cost of diabetes-related healthcare. Home testing by diabetics will undoubtedly decrease the cost of diabetes even further as fewer numbers of doctors' visits will be necessary. Thus, the method of choice for blood glucose control would be self-determination of one's own blood glucose level using a glucose biosensor.

Therefore, it is of no surprise that currently, the development of a successful glucose biosensor is one of the most financially attractive areas in medical diagnostics. The various studies have proven that frequent monitoring can reduce the cost of diabetes-related healthcare. The increase in healthcare costs around the world necessitates the introduction of cost-effective, diagnostic glucose-testing kits. In addition, human lifespan in many developed countries has increased, resulting in a substantial aging population, which leads to a rise in the incidence of type 2 diabetes.

These are among the factors behind the need for continuing research in the area of blood glucose monitoring.

Over the years, a wide range of methods for measuring blood glucose concentration has been studied. The first ones to materialize were glucose assays based on glucose oxidase and reagents impregnated in paper strips, which give diabetics a qualitative indication of their blood glucose level. Then, the paper strips were combined with a reflectometer [5].

Another method employs the concept of glucose concentration determination using amperometric techniques. Glucose concentration can be measured by electrochemically following the decrease in oxygen concentration as the reaction proceeds [6-7], the production of hydrogen peroxide [8-10] or the change in pH with the production of D-gluconic acid. The oxygen and hydrogen peroxide based glucose sensors are the so-called first generation amperometric glucose sensors. Second generation glucose sensors make use of mediators to shuttle electrons from the enzyme to the electrode [11-13]. Third generation amperometric glucose sensors are based on the use of conducting organic salts or polymers. The films are grown electrochemically and glucose oxidase is entrapped in the membranes. Polypyrrole [14] and polyaniline [15] have been investigated as potential membrane materials for the entrapment of the enzyme. To date, however, amperometric glucose biosensors based on hydrogen peroxide continue to dominate the field of glucose sensor research due to its simplicity.

An important consideration in the practical application of glucose biosensors is the operational life of the sensing element. Considerable research effort has been focused on the method of immobilizing glucose oxidase since the technique employed may influence the available activity of the enzyme and thus affect the performance of the sensor. However, narrow measuring range and low current response, which are a direct consequence of the effectiveness of the immobilized

glucose oxidase, are still considered a problem. Most of these problems stem from conformational changes that occur when the enzyme is being immobilized.

Some of the conventional methods of immobilization that have been investigated include covalent attachment to a reactive insoluble support, physical adsorption to a solid surface, physical entrapment in polymeric gels, and cross-linking with a bifunctional agent such as glutaraldehyde, often in combination with adsorption or physical entrapment [16]. Newer approaches include the entrapment of glucose oxidase in electrochemically grown polymeric films such as polyaniline and polypyrrole [17]. GOD has been immobilized on methacrylate copolymers [18], polyaniline [19,20], poly(phenol) films [21], poly(vinyl ferrocene) films [22], ferrocenyl-acrylamide-acrylic acid copolymer films [23], polypyrrole films [24] and cellulose acetate [25].

In this project, various methods of glucose oxidase immobilization for peroxide based amperometric glucose biosensor were investigated. Numerous parameters (such as temperature, concentration, etc.) that can affect the activity of the enzyme were considered in order to determine the optimized method for GOD immobilization. The "old school" method of changing one control variable at a time was employed for the optimization.

1.2 Objective

The objective of this project was to examine various ways to improve the performance of immobilized glucose oxidase membranes for peroxide based amperometric glucose biosensors.

1.3 Scopes

The scopes for this project were as follows:

a) Immobilization of glucose oxidase using different methods. Various immobilization parameters were investigated.

b) Determination of the performance of the immobilized glucose oxidase membrane. Various characteristics of the membrane were investigated.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Biosensor

Biosensor history started in 1962 when American Scientist Leland C. Clark studied the electrochemistry of oxygen reduction at a platinum metal electrode, pioneering the use of an oxygen sensor [26]. The electrode was eventually coined a "Clark Electrode". Clark then placed entrapped glucose oxidase next to the platinum electrode and followed the activity of the enzyme by following the changes in oxygen concentration, thereby turning the chemosensor into a glucose biosensor.

The first step towards commercial exploitation was taken by the Yellow Springs Instrument Company (YSI) in the 70s. YSI collaborated with Clark to develop a series of laboratory-scale glucose sensors. A lot of work went into finding suitable membrane that rendered the GOD-platinum electrode technique reproducible and accurate.

Today, mass marketing of home-monitoring glucose systems has become a reality. At present, most of the home-monitoring glucose systems on the market are enzyme-photometric or amperometric methods and are of the *in vitro* type. However,

the commercial market is changing rapidly. Cygnus GlucoWatch Biographer [27], a minimally invasive glucose sensing system based on iontophoresis, and the Minimed Continuous Glucose Monitoring (CGM) system [28], a minimally invasive transcutaneous glucose sensor, have received U.S. Food and Drug Administration (FDA) approval.

2.2 Physical Principles of glucose sensing

Over the years, a wide range of methods for measuring blood glucose concentration has been studied. The first ones to materialize were glucose assays based on glucose oxidase and reagents impregnated in paper strips. When blood is placed on the paper strip, hydrogen peroxide that is produced from the enzymatic reaction oxidizes an oxygen acceptor in the presence of peroxidase to form a color change. The intensity in the color of the paper strips will give the diabetics a qualitative indication of their blood glucose level. However, the qualitative nature of the tests prompted researchers to turn to other approaches that can provide more quantitative results. One approach is to combine the use of the paper strips with a reflectometer. When light is shined on the test pad, it will be reflected differently according to the intensity of the color of the test strip. The reaction reflectance will be measured electronically and a blood glucose concentration value is displayed. Systems using absorbance photometry utilize two wavelengths to measure the reaction reflectance instead of the single wavelength used by most reflectance photometry systems [5]. In recent versions of the reflectance-based method, apart from the glucose oxidase-peroxidase-dye optical method, glucose oxidase has also been coupled with other reagents such as the glucose oxidase-prussian blue method, glucose oxidase-organic mediator optical method and others. Some systems use hexokinase instead of glucose oxidase for glucose detection [29].

Another method employs the concept of glucose concentration determination using amperometric techniques. The basic underlying chemistry is the catalytic action of glucose oxidase, which oxidizes D-glucose according to the following reaction:

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-gluconic acid} \quad (2.1)$$

The reaction will become rate limited if either glucose or oxygen concentration is too low. Glucose concentration can be measured by following the decrease in oxygen concentration as the reaction proceeds, the production of hydrogen peroxide or the change in pH with the production of D-gluconic acid electrochemically. To date, the most widely researched methods of detection are the monitoring of oxygen or hydrogen peroxide at an electrode.

For the oxygen-based enzyme glucose sensor [6-7], since oxygen is consumed during the enzymatic reaction, oxygen concentration in the glucose oxidase membrane will be a linear function of glucose concentration. The oxygen concentration can be detected by coupling the membrane containing immobilized glucose oxidase to an electrochemical oxygen sensor. Since oxygen is also present in the sample, a similar reference oxygen sensor without the enzyme needs to be incorporated in the system. The signal current is then subtracted from the reference electrode and this results in a glucose-dependent difference current. The advantages of this type of sensor is that it has low electrochemical interference due to the use of a nonporous hydrophobic membrane that will only allow gaseous molecules to reach the electrode and it can also provide information on oxygen variations in the system. Furthermore, the immobilization of the enzyme catalase along with glucose oxidase will help prolong the active lifetime of glucose oxidase as catalase promotes the degradation of hydrogen peroxide to oxygen and water. However, the differential set-up makes it a more complicated device and renders it hard to miniaturize.

The hydrogen peroxide-based enzyme glucose sensor [8-10] has found wide application in the development of a glucose biosensor, especially an implantable

version, due to its simple sensor configuration that facilitates ease of miniaturization. Unlike oxygen, hydrogen peroxide is not present in the sample to be analyzed, so no differential set-up is needed. However, it suffers from an intrinsic problem, the interference from small endogenous analytes such as urate and ascorbate and some common drugs such as acetaminophen. These species are electro-active at the detection potential of hydrogen peroxide.

The interfering effects of most of the endogenous species have been shown to be effectively eliminated by the use of a number of internal membranes such as cellulose acetate[30] or Nafion® [31]. Since these membranes impart negative charges by the presence of residual carboxyl groups or sulphonate groups, the transport of anionic species such as ascorbate and urate can be easily retarded. However, since acetaminophen is an uncharged molecule, it can still cause a considerable bias in the sensor output even with these membranes. A number of studies have reported that the interference caused by acetaminophen can be reduced by using composite membranes such as cellulose acetate and Nafion®[32]; and γ-aminopropyltriethoxysilane , cellulose acetate and Nafion® [33].

The oxygen and hydrogen peroxide based glucose sensors are the so-called first generation amperometric glucose sensors. Second generation glucose sensors make use of mediators to shuttle electrons from the enzyme to the electrode [11-13]. This type of system is supposed to eliminate the dependency of the enzymatic reaction to oxygen. If the system is oxygen deficient, the glucose sensor will become insensitive to glucose and will only respond to changes in oxygen concentration. However, as oxygen remains in the system, the mediator must be able to compete effectively for the electrons. Ferrocene and its derivatives are the best-known artificial electron carriers.

If the enzyme membrane contains both ferrocene and glucose oxidase, the following reaction will occur:

$$\text{Glucose} + 2\ \text{ferricinium}^{+} + H_2O \xrightarrow{\text{Glucose oxidase}} \text{gluconic acid} + 2\ \text{ferrocene}. \qquad (2.2)$$

At a positively biased electrode, this reaction follows:

$$2\ \text{ferrocene} \longrightarrow 2\ \text{ferricinium}^{+} + 2e^{-}. \qquad (2.3)$$

Glucose sensors based on this principle can display a linear range up to 30mM even when oxygen concentration in the system is very low. The advantage of this system is that the oxidation of the mediator can be carried out at a significantly lower potential than that of hydrogen peroxide resulting in lower electrochemical interference from endogenous species. However, the mediators can be toxic, thus eliminating the possibility of *in vivo* use. Heller and his group [34] have designed a recent version of the mediator sensor, which is not diffusable. The mediator is bound to a cross-linked polymer. Glucose oxidase is tethered to the electrode with a hydrogel formed of a redox polymer with a bound complexed osmium redox center.

Third generation amperometric glucose sensors are based on the use of conducting organic salts or polymers. The films are grown electrochemically and glucose oxidase is entrapped in the membranes. Polypyrrole [14] and polyaniline [15] have been investigated as potential membrane materials for the entrapment of the enzyme. The electrode responds to glucose concentration via peroxide oxidation. The advantage of this system is that manipulation of the electropolymerization can

give a film that extends the linear range for glucose detection and reduces oxygen dependence.

Another concept that is rapidly gaining interest is the fluorescence-based optical glucose affinity sensor. Glucose detection is via competitive displacement of a fluorescently-labeled competing sugar ligand from an immobilized protein with binding sites for certain carbohydrates. With increasing glucose concentration, the labeled competing sugar ligand will be displaced from the immobilized protein causing it to diffuse freely into the volume illuminated by a light source. This will lead to a glucose concentration-related increase in fluorescence. The concept was first developed using concanavalin A as the protein and high molecular weight fluorescein labeled dextran as the competing sugar ligand [35]. As a substitute to the single fluorophore technique, both the protein and the competing ligand can be labeled. Meadows, et al. [36] labeled high molecular weight dextran and concanavalin A with fluorescein isothiocyanate and tetramethylrhodamine isothocyanate, respectively. Two optical fibers were used with one employed to detect fluorescein isothiocyanate emission to determine glucose concentration and the other one employed to detect tetramethylrhodamine isothocyanate emission to account for drift and changes in configuration.

In recent years, the concept of non-invasive optical glucose sensors has also garnered attention. Light in the near infrared or other region of the spectrum is beamed on to a relatively transparent part of the body such as an ear lobe or finger web [37]. Glucose absorbs near infrared light in the 1000 to 2500 nm region where skin and tissue are non-absorbing. The light signal is processed by mathematical filtering techniques to maximize the signal that can correlate with blood glucose concentration. This concept has also been attempted with the technique of attenuated total reflectance infrared spectroscopy; however, the penetration depth is much smaller than that of the near infrared light [38]. Although the concept of an optical non-invasive sensor is appealing, the lack of adequate selectivity is a major problem. Other biological species in the area along with tissue structures can interfere with

glucose measurement. The systems also suffer from complexity in obtaining accurate calibration as the correlation between signal and blood glucose can change with time, thus rendering it useless for real time monitoring.

Several approaches to non-invasive transdermal extraction of tissue fluids have also been considered as substitutes to blood sampling. Even though this method does not make use of any new working principle of glucose sensing, the fact that it can provide an attractive alternative to frequent blood collection makes it worth mentioning. This method is also termed the minimally-invasive technique of blood glucose measurement by some. Tissue fluid can be extracted by means of suction or reverse iontophoresis [39, 40]. In the reverse iontophoresis method, an externally applied potential is used to promote molecular transport through the skin. The transdermally-extracted samples can be analyzed using any type of glucose sensing technique *ex vivo*. The samples seem to be less aggressive to the sensor than blood, thus minimizing biofouling. However, there are some points that still need clarifying such as long term effects of current on skin; lag in response time as fluid collection might require at least 15 minutes; the possibility of inconsistency in glucose recovery at different sites and the frequency of sensor recalibration.

The description above is not intended to be a comprehensive summary of all the physical principles used for glucose determination. Among those that are not mentioned here are potentiometric glucose electrodes [41], sensors based on microcalorimetry [42] and others [43, 44].

2.3 Glucose Oxidase (GOD)

Various methods of glucose sensing have been discussed in detail in part 2.2. One popular method involves the use of glucose oxidase (GOD). GOD is a highly specific enzyme for D-glucose, from the fungi *Aspergillus Niger* and *Penicillium*, which catalyses the oxidation of β-glucose to glucono-1,5-lactone, which spontaneously hydrolyses in the absence of enzyme to gluconic acid using molecular oxygen or artificial electron acceptor.

GOD is a dimeric protein as shown in figure 2.1 with a molecular weight of 160, 000 Dalton. It contains one tightly bound flavin adenine dinucleotide (FAD) per monomer as cofactor which means that each enzyme will have two FAD-sites. FAD can be released from the protein following partial unfolding of the protein since it is not covalently bound. FAD in GOD is shown in figure 2.2.

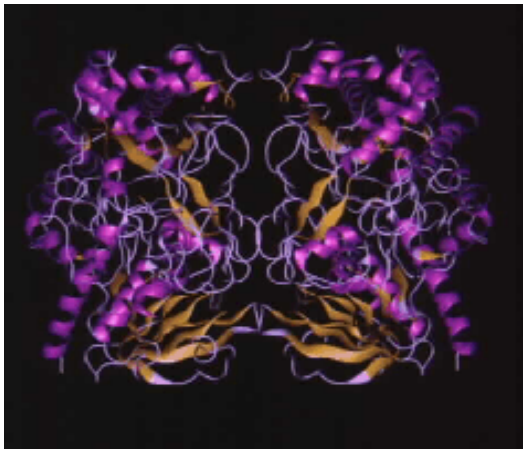

Figure 2.1 Overall topology of Glucose oxidase.

GOD is made up of two identical subunits with a molecular weight of 80,000 Dalton each. These two monomers are connected non-covalently via a long but narrow contact area. There are either salt linkages or hydrogen bonds between the dimers. As shown in figure 2.2, the monomer folds into two structural domains.

One of the domains involved with substrate binding and the other binds FAD. The corresponding dimensions of the dimer are 70 Å x 55 Å x 80 Å. Properties of GOD are shown in table 2.1.

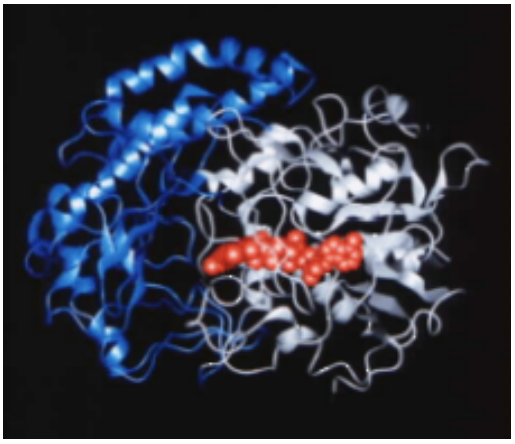

Figure 2.2 Subunit structure of GOD showing FAD (red spacefill)

Table 2.1: General characteristics of glucose oxidase

Characteristics	Description
Molecular weight	160, 000 Dalton
Composition	The enzyme consists of two identical polypeptide chain subunits (80, 000 Dalton) covalently linked by disulfide bonds. Each subunit contains one mole of Fe and one mole of FAD. The molecule is to be approximately 74% protein, 16% neutral sugar and 2% amino sugars. It is indicated that the FAD is replaceable with FHD (Flavin-Hypoxanthine Dinucleotide) without loss of activity.
Optimum pH	5 with broad range 4-7
Specificity	The enzyme is highly specific for β-D-glucose. The α anomer is not acted upon. 2-deoxy-D-glucose, D-mannose and D-galactose exhibit low activities as substrate.

Inhibitors	Ag+, Hg2+, Cu2+. FAD binding is inhibited by several nucleotides.
Stability	Dry preparations are stable for years when stored cold. Solutions are reasonably stable under a variety of conditions.

2.4 Immobilization of Enzymes

Enzyme immobilization is defined as the restriction of enzyme mobility in a fixed space. In order to make a viable biosensor, the biological component (enzyme) has to be properly attached to the transducer. Design, preparation procedure and immobilization procedures is the key to construct a successful biosensor so that enzymes should be stabilized and easy to be contacted by substrates. Choice of immobilization technique to immobilize enzyme is extremely important in terms of biosensor operational stability and long-term use. Methods selected must be compatible with the enzyme and substrates. Poor technique will result in significant loss of enzyme activity and thus low sensor response. A critical step in fabrication of these devices is effective enzyme immobilization while maintaining free diffusion of the substrates to the enzyme layer.

2.4.1 Entrapment

Entrapment is a physical method to immobilize or physical enclosure of enzymes in a small space. Enzyme remains free in the solution, but restricted in movement by the lattice structure of a gel. Pore size of the gel lattice is controlled so that the structure is tight enough to prevent enzyme leakage while allowing free movement of substrates and products. This method is applicable to many enzymes, may provide no or little perturbation of the native structure and function of the

enzyme, very selective to the enzyme sensed and is a good method to trap microorganisms. Some of the problems associated with entrapment are due to the loss of enzymatic activity and large response time.

Matrix entrapment and membrane entrapment; including microencapsulation are the two major methods of entrapment. The matrix can be a particle, a membrane or a fiber [45]. Figure 2.3 shows the entrapment of enzyme into a gel or polymer network.

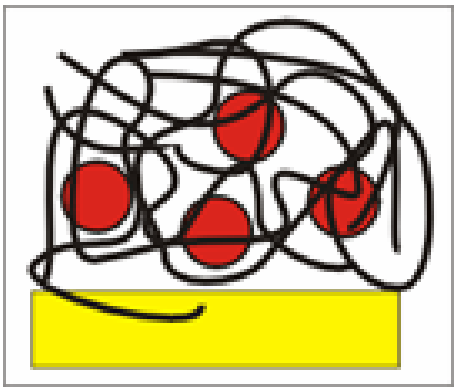

Figure 2.3 Entrapment into a gel or polymer network.

A special form of membrane entrapment is microencapsulation. The main types of membrane used are cellulose acetate, polycarbonate, collagen, polytetrafluoroethylene (TEFLON), nafion and polyurethane. This is the method used in the early biosensors such as Clark type sensors. Microscopic hollow spheres are formed and it contains the enzyme solution while being enclosed within a porous membrane. There is a close attachment between the enzyme and the transducer and it gives a good stability to changes in temperature, pH, ionic strength and substrate concentration.

2.4.2 Adsorption

In adsorption, enzyme is attached on the surfaces of support/carrier particles by weak but sufficiently large forces to allow reasonable binding forces such as van der Waals, ionic and hydrogen bonding interaction and possibly hydrophobic forces as illustrated in figure 2.4. This method is only suitable for exploratory work over short periods of time. This method may be stabilized by cross-linking of glutaraldehyde but this might denature some of the proteins. Many substances adsorb enzymes on their surfaces but the surfaces of the support materials may need to be pretreated either chemically or physically for effective immobilization. The advantages of choosing adsorption as the immobilization technique are that usually no reagents are required and involve minimal preparation and clean up work. Nearly full activity of the enzyme is retained since the active site of the adsorbed enzyme is unaffected. Despite its simplicity, adsorbed enzymes are susceptible to ambient conditions such as pH, temperature, ionic strength, polarity etc which will cause leakage of enzymes from supports. Another disadvantage is that there is no specific binding by substrate or contaminants to the carrier which may result in diffusion limitations and mass transfer problems.

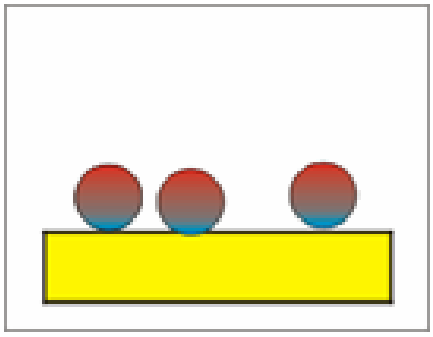

Figure 2.4 Adsorption to the surface.

2.4.3 Covalent Binding

Covalent binding is the retention of enzymes on support surfaces through the formation of a covalent bond between functional group on the carrier and the enzyme as illustrated in figure 2.5. Those on enzymes are usually amino acid residues such as amino group from Lysine or Arginine, carboxyl group from Aspartic acid and Glutamic acid, hydroxyl group from Serine and Threonine and sulfhydryl groups from Cystine. Formation of covalent bonds must not inactivate the enzyme. This is ensured by blocking the active site by flooding the enzyme solution with a competitive inhibitor prior to covalent binding.

This mode of attachment often involves three steps including the activation of the support, the modification of the activated electrode surface and the enzyme coupling. Reactions have to be carried out at mild temperature and low pH. This method is widely applicable since it provides a more stable immobilized biomolecule layer and modification with more than one layer is possible. Besides, the enzyme is permanently attached to the support.

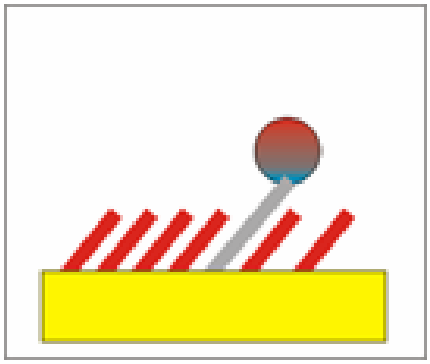

Figure 2.5 Covalent linkage to the electrode or a self-assembled monolayer .

2.4.4 Cross-linking

This method joins the enzyme to each other or to another supporting material to form a large 3D structure. This can be done by using bi-functional agents such as gluteraldehyde, bis-diazobenzidine and 2,2-disulfonic acid. In cross-linking, enzymes can be cross-linked with glutaraldehyde to form an insoluble aggregate, adsorbed enzymes may be cross-linked or cross-linking may take place following the impregnation of porous support material with enzyme solution. Cross-linking is a useful method to stabilize adsorbed enzyme. It may cause significant changes in the active site of enzymes and thus, tests must be done to ensure that the active site remains free and available for catalytic activity. It may result in severe diffusion limitations and poor mechanical strength or rigidity.

2.5 Limitations Associated with Immobilization Methods

With immobilized enzymes, reaction rates depend not only on substrate concentration and kinetic constants but also on immobilization effects [46]. These effects are due to the alteration of the enzyme by the immobilization process.

2.5.1 Conformation

Conformation changes of the enzyme caused by immobilization usually decrease the affinity to the subtrate (increase Michaeles constant, K_m). Furthermore, a partial inactivation of all, or the complete inactivation of a part of the enzyme molecules may occur (decrease of maximum velocity, V_m).

2.5.2 Microenvironment

Ionic, hydrophobic or other interaction between the enzyme and the matrix (microenvironmental effects) may also result in changed K_m and V_m values. These essentially reversible effects are caused by variations in the disassociation equilibria of charged groups of the active center.

2.5.3 Non-uniform Distribution

A non-uniform distribution of substrate and/or product between the enzyme matrix and the surrounding solution affects the measured (apparent) kinetic constants.

2.5.4 Reaction and Diffusion

In biosensors, the enzyme reaction proceeds in a layer separated from the measuring solution. The substrate reaches the membrane system of the biosensor by convective diffusion from the solution. The rate of this external transport process depends essentially on the degree of mixing. In the multilayer system in front of the sensor, the substrates and products transferred by diffusion. Slow mass transfer to and within the enzyme matrix leads to different concentrations of the reaction partners in the measuring solution and in the matrix. Diffusion and the enzyme reaction do not proceed independently of one another. They are coupled in a complex manner.

2.6 Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA) is manufactured by the hydrolysis of polyvinyl acetate. Even after a prolonged hydrolysis, PVA generally retains around 1 to 2 mole percent of acetate groups. The amount of residual acetate groups affects the physical and chemical properties of PVA, as they are hydrophobic relative to the hydroxyl groups. The residual acetate groups can interfere with inter-molecular and intra-molecular forces such as hydrogen bonding. Highly hydrolyzed PVA has strong hydrogen bonds within and between molecules [47].

PVA, unlike many polymers, is soluble in water. However, owing to strong internal hydrogen bond, it only goes into solution at higher temperatures, around 90^0C. Aqueous solutions of PVA exhibit non-Newtonian behavior at room temperature [48]. PVA can be cross-linked chemically or physically to form a hydrogel. Chemically, the polymer can be cross-linked by any bifunctional agent that can react with organic hydroxyl groups. Some of the various chemicals that can cross-link PVA are glutaraldehyde, formaldehyde, maleic acid and boric acid. Cross-linking can also be achieved physically using ultraviolet light in the presence of photo-sensitizers, by electron beam or by γ-radiation. Another physical method of cross-linking PVA is through freeze thaw cycles, where physical bonds are formed [49].

Cross-linking causes PVA to be insoluble in water. Furthermore, by controlling the cross-link density of this material, a variety of transport properties can be obtained. In addition, poly(vinyl alcohol) is also considered biocompatible. Protein adsorption onto cross-linked PVA has been shown to be negligible, thus the potential of minimizing fibrotic capsule development around implantable PVA exists [50]. Hence, due to its promised biocompatibility, ease of manipulation and hydrophilicity, PVA has been used extensively in biomedical applications. In the biomedical area, PVA has found use in hernia treatment [51], in artificial heart valve

replacement [52] and as a drug carrier in controlled drug release system [53], among others.

In dense hydrogels, diffusion of solutes is determined by the cross-linking density of the network, or mesh size, and the degree of swelling. The "pores" are the open spaces between the cross-link points and are not fixed. Different theories have been proposed to describe solute diffusion in hydrogels. Polymer chains are hypothesized to hinder solute diffusion through one or a combination of these methods: by physically obstructing the passage of solute, by increasing the hydrodynamic drag on the solute molecule or by reducing the available free volume for the solute [54].

Transport properties of cross-linked of PVA has been studied extensively. Korsmeyer and Peppas [55] examined the correlation between the degree of cross-linking and hydration, on the diffusion of drugs in PVA hydrogels ; Reinhart and Peppas [56] studied the influence of the degree of cross-linking of PVA hydrogels on the diffusion of bovine serum albumin (BSA) ; Li and Barbari [57, 58] investigated protein transport through surface modified poly(vinyl alcohol) hydrogels; Dai and Barbari [59, 60, 61] studied the transport properties of poly(vinyl alcohol) with mesh size asymmetry based on gradient cross-linking with glutaraldehyde and examined the possibility of using the modified PVA for cell encapsulation or bioseparations; and Hideto et al. [62] analyzed the diffusion of solutes with molecular weights ranging from 180-66,000 in cross-linked PVA with free volume theory. The various properties of PVA have made them suitable for enzyme immobilization.

2.7 Sol-gel

The sol-gel process involved the initial hydrolysis and polycondensation of suitable precursors to form ceramic materials, leading to the formation of colloidal particles, which is called sol. The complex reaction is often catalyzed by either an acid or base [63]. As the interconnection between these particles increases, the viscosity of the sol starts to increase and this leads to the formation of the porous gel, which can be used as enzyme encapsulation matrix. When dried near room temperature, the dried sol-gel matrix provides an aqueous environment inside the pores, which made it suitable as a host for the enzymes. Due to the porous nature of the matrix, an analyte can interact easily with immobilized enzyme. The porous inorganic sol-gel matrix possesses physical rigidity, chemical inertness, high photochemical, biodegradational, tuneable porosity, and experiences negligible swelling in both aqueous and organic solutions.

Cracking can easily occur due to capillary stresses generated by evaporation of water and solvent molecules from the porous network. Slower hydrolysis occurs under acidic condition creating a polymeric gel with a smaller average pore size, which may lead to diffusional restraints in the sol-gel matrix, resulting in a lower initial enzyme activity but more rigid enzyme layer.

$$\equiv Si{-}OR + H_2O \underset{}{\overset{\text{acid or base}}{\rightleftharpoons}} \equiv Si{-}OH + ROH \qquad \text{Hydrolysis} \qquad (2.4)$$

$$\left.\begin{array}{l} \equiv Si{-}OH + HO{-}Si\equiv \rightleftharpoons \equiv Si{-}O{-}Si\equiv + H_2O \\ \equiv Si{-}OR + HO{-}Si\equiv \rightleftharpoons \equiv Si{-}O{-}Si\equiv + ROH \end{array}\right\} \text{Condensation} \qquad (2.5),\ (2.6)$$

In a typical procedure, tetramethoxysilane (or tetraethoxysilane) is mixed with water in a mutual solvent such as methanol followed by the addition of suitable catalyst. As the sol becomes interconnected, a macroscopically rigid, hydrated gel is formed. Specific reagents such as enzymes can be trapped into this optically transparent,

stable host matrix by simply adding them to the sol prior to its gelation. Such molecules become entrapped in the growing covalent gel network rather than being chemically bound to the water-rich inorganic matrix, so that enzyme activity can be maintained. These materials have been used in numerous applications including solid-state electrochemical devices, chemical sensors, catalysts, and nonlinear and optic applications.

Ratio of TMOS and water of the stock sol-gel solution is one of the most significant process parameters for controlling the pore size of the matrix. An *R* value, which is the water/alkoxide ratio, of 1:3.7 was seen to be optimal [64]. Higher *R* value causes increase in the rate of hydrolysis resulting in a more particulate gel.

Different alkoxides may give different properties to the resulted sol-gel matrix. Two types of alkoxides are usually applied, the silica alkoxide, tetramethoxysilane (TMOS) ($C_4H_{12}O_4Si$), and metal alkoxides, alumina (aluminium isopropoxide)($Al[OCH(CH_3)_2]_3$). .

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Glucose oxidase (EC 1.1.3.4, type X-S, 157,500 units/g solid) from *Aspergillus niger* and horseradish peroxidase (EC 1.11.1.7, type VI from horseradish, 330 purpurogallin units/mg solid) were purchased from Sigma.(3-glycidoxypropyldimethylethoxy)silane was purchased from Fluka. Poly (vinyl alcohol) with average molecular weight of 70,000 – 100,000, O-dianisidine tablets (10 mg substrate/tablet) and glutaraldehyde (1.2% w/v aqueous solution) were obtained from Sigma Chemical Co. Tetramethoxysilane (TMOS), sodium potassium tartrate tetrahydrate, D-glucose monohydrate, copper sulfate pentahydrate, sodium hydroxide, potassium dihydrogen phosphate (KH_2PO_4) and potassium hydrogen phosphate (K_2HPO_4) were obtained from MERCK. All chemicals were used as received.

3.2 Instrumentation

Electrochemical measurements were carried out using a Metrohm μ Autolab Type 111 potentiostat. A conventional three-electrode electrochemical cell was employed. A platinum electrode was used as the working electrode (WE), a platinum auxiliary electrode was used as the counter electrode (CE) and a Ag/AgCI/KCI was employed as the reference electrode (RE). Colorimetric measurements were done using a Hitachi V-100 UV spectrophotometer.

3.3 GOD immobilizations methods

3.3.1 GOD immobilization in cross-linked PVA matrix: different casting temperature

25μL of 10 wt% aqueous PVA solution was mixed with a cross-linking solution, which consisted of 15μL 10% v/v acetic acid (buffer), 10μL 50% v/v methanol (quencher), 5μL 10% v/v sulfuric acid (catalyst) and 28μL of 1.2 % w/v glutaraldehyde, so that it ended up with a cross-linking ratio (CR) of 0.06. Cross-linking ratio is defined as the ratio of the moles of glutaraldehyde per mole of PVA repeat unit.

36μL of the solution was added to 6μL GOD solution (200 mg/mL). An aliquot of the mixture was pipetted quickly onto a glass slide, air-dried for 20 minutes and covered with another glass slide. The two glass slides were clamp together, to prevent the membrane from contracting, and left for either 2 days at 4°C

or 24 hours at 25°C. Membrane thickness was controlled with aluminium spacer tape where 3 layers of aluminium spacer tape were stuck on the first glass slide.

Following the polymerization, the slides were soaked in 35mL of phosphate buffer, pH 6.0 for a prescribed amount of time at room temperature in a petri dish. The slides were unclamped carefully and the layer was gently stripped from the slide. The washing solutions were collected for enzyme assay purposes and the membranes were swollen in 5mL of phosphate buffer at 4°C. The buffer was changed every 6 hours for the first day, every 12 hours for the second day, and 24 hours thereafter, and analyzed for any sign of enzyme activity.

3.3.2 GOD immobilization in cross-linked PVA matrix: different PVA concentration

25μL of either 10 wt% or 15 wt% aqueous PVA solution was mixed with a cross-linking solution, which consisted of 15μL 10% v/v acetic acid (buffer), 10μL 50% v/v methanol (quencher), 5μL 10% v/v sulfuric acid (catalyst) and 28μL of 1.2 % w/v glutaraldehyde, so that it ended up with a cross-linking ratio (CR) of 0.06. Cross-linking ratio is defined as the ratio of the moles of glutaraldehyde per mole of PVA repeat unit.

36μL of the solution was added to 6μL GOD solution (200 mg/mL). An aliquot of the mixture was pipetted quickly onto a glass slide, air-dried for 20 minutes and covered with another glass slide. The two glass slides were clamp together, to prevent the membrane from contracting, and left for 24 hours at 25°C. Membrane thickness was controlled with aluminium spacer tape where 3 layers of aluminium spacer tape were stuck on the first glass slide.

Following the polymerization, the slides were soaked in 35mL of phosphate buffer, pH 6.0 for a prescribed amount of time at room temperature in a petri dish. The slides were unclamped carefully and the layer was gently stripped from the slide. The washing solutions were collected for enzyme assay purposes and the membranes were swollen in 5mL of phosphate buffer at 4°C. The buffer was changed every 6 hours for the first day, every 12 hours for the second day, and 24 hours thereafter, and analyzed for any sign of enzyme activity.

3.3.3 GOD immobilization in freeze-thawed PVA matrix

60µL of PVA solution (5%, 10% or 15% w/v) was added to 10µL of 280 mg/ml GOD solution. An aliquot of the mixture was pipetted quickly onto a polymer block and covered with another polycarbonate block. The two blocks were clamped together in order to prevent the membrane from contracting. The membrane was then left to freeze at -20^0C for 12 hours. After freezing the membrane was thawed at 4^0C for 12 hours. The process was repeated 5 times. The membrane thickness was controlled with aluminum spacer tapes.

After the freezing and thawing process, the blocks were soaked in 40 ml of phosphate buffer, pH 6.0 at room temperature. The blocks were slowly unclamped and the membrane was gently stripped from the block. 1mL of the washing solution was collected for enzyme assay purposes. The buffer was changed every 6 hours for the first day, every 12 hours for the second day and 24 hours thereafter and analyzed for any sign of enzyme activity.

3.3.4 GOD immobilization in PVA/TMOS sol gel matrix

TMOS, (3-glycidoxypropyldimethylethoxy)silane, H_2O, methanol and 57.5μL of HCI were mixed together, in a volume ratio of 1 : x : 3.7 : 3.7 : 0.0013, where x was 1, 2 and 3 respectively. The solution was stirred for 30 minutes. 115 μL of HCI was later added and the sol gel solution was stirred for 1 hour. 25μL of PVA solution was mixed with 100μL of the sol gel solution. 90μL of the solution was then added to 15μL of 140 mg/ml GOD solution. An aliquot of the mixture was pipetted quickly onto a glass slide. The membrane was air-dried for 20 minutes and then covered with another glass slide. The two glass slides were clamped together to prevent the membrane from contacting and left for 24 hours at 25ºC. Membrane thickness was controlled with aluminium spacer tapes.

After 24 hours, the slides were soaked in 25mL phosphate buffer solution in a petri dish at 25ºC. The slides were carefully unclamped and the membrane layer was gently stripped from the slide. The membrane was swollen in 5mL phosphate buffer solution and kept at 4ºC. 1mL of the washing solution was collected for enzyme assay purposes. The buffer was changed every 6 hours for the first day, every 12 hours for the second day and 24 hours thereafter and analyzed for any sign of enzyme activity.

3.4 Measurement of enzyme activity in washing solution

Enzyme activity in the washing solution was measured using either BCA method or Biuret method.

3.4.1 BCA method

A Standard working solution (SWR) was prepared by mixing reagent A (0.04 g/mL $CuSO_4.5H_2O$) and reagent B (0.01 g/mL BCA, 0.02 g/mL $Na_2CO_3.H_2O$, 0.0016 g/mL $Na_2C_4H_4O_6.H_2O$, 0.004 g/mL NaOH and 0.0095 g/mL $NaHCO_3$) in a ratio of 50:1.

Bovine Serum Albumin (BSA) was used as the protein standards. The concentrations used were from 0 mg/mL – 1.0 mg/mL. 2 mL of SWR was added into each test tubes that contained 0.1 mL of either the BSA solution or the washing solution. The tubes were incubated at 37^0C for 10 minutes. The absorbance for the standards and unknowns were recorded at 450 nm.

3.4.2 Biuret method

6.0g sodium potassium tartrate tetrahydrate ($NaKC_4H_4O_6.4H_20$) was dissolved in 500mL distilled water. 1.5g of copper sulfate pentahydrate ($CuSO_4.5H_2O$) was then added and dissolved into the solution. After that, 300mL of 10%w/v sodium hydroxide (NaOH) was added slowly with stirring. Finally, the solution was diluted to 1L with distilled water. The solution was stirred until homogenous. The Biuret reagent was stored in a Schott® bottle covered with aluminium foil and kept in shaded place for subsequent use due to its sensitivity to light.

Bovine Serum Albumin (BSA) was used as the protein standards. The concentrations used were from 0 mg/mL – 12.5mg/mL. For the protein assay, 3 mL of biuret reagent was added into each test tube that contained 2 mL of either BSA

solution or the washing solution. Then the tubes were incubated at 37 ºC in a water bath for 15 minutes. The absorbance of each solution was measured against a blank (biuret solution without BSA) at a wavelength of 550nm.

3.5 Measurement of immobilized enzyme apparent activity

Immobilized enzyme apparent activity was measured using either colorimetric method or amperometric method.

3.5.1 Colorimetric method

The chromogen solution was prepared by diluting 0.1mL of 1% w/v O-dianisidine in 12mL of phosphate buffer at pH 6.0. 450μL of 25%w/v of aqueous glucose solution (allowed to mutarotate overnight) and 150μL of 200μg/mL peroxidase were added to 3.75mL of the chromogen solution. The mixture was incubated at room temperature for temperature equilibration. For enzyme activity calibration curve, 150μL of GOD with the activity ranging from 0 to100 mU/mL was mixed with the mixture. For apparent immobilized enzyme activity determination, the membrane was dipped in a 5mL universal bottle and the solution was stirred. For both sets of experiments, reaction was allowed to proceed at room temperature for 10 minutes. 300μL of 4M HCl was added to stop the reaction. However, for the apparent enzyme activity assay, the membrane was removed prior to the addition of the acid. The amount of colour formed was measured by reading the absorbance value at 450nm. Apparent enzyme activity was determined using a standard

calibration curve. One unit of activity causes the oxidation of one micromole of O-dianisidine per minute at 25°C and pH 6.0 under the conditions specified.

3.5.2 Amperometric method

For amperometric experiments, a conventional three-electrode electrochemical cell was employed. A platinum electrode was used as the working electrode (WE), a platinum auxiliary electrode was used as the counter electrode (CE) and a Ag/AgCI was employed as the reference electrode (RE).

The enzymatic membrane was attached to the surface of the working electrode. Before used, the electrode was rinsed with deionized water and immersed in 15mL of phosphate buffer with pH 7.0. Voltage at 0.7 V vs Ag/AgCl was applied to the system. The electrochemical response was let to stabilize. Freshly prepared enzymatic membranes could attain a stable electrochemical response after 5 to 10 minutes of rinsing. After the current had stabilized, a prescribed amount of stock glucose solution was injected into the cell to make up a 5 mM glucose solution. The change in current is proportional to the apparent enzyme activity immobilized in the membrane. The amperometric studies were performed at 25^{o}C.

3.6 Determination of enzyme kinetics

The enzymatic membrane was attached to the surface of the working electrode. Before used, the electrode was rinsed with deionized water and immersed

in 15mL of phosphate buffer with pH 7.0. Voltage at 0.7 V vs Ag/AgCl was applied to the system. The electrochemical response was let to stabilize. Prescribed amount of stock glucose solutions were injected sequentially to the electrochemical cell to make up a variety of glucose solution concentrations. Upon each addition of glucose solution, the current was let to stabilize before the next glucose injection. All experiments were performed at a temperature of 25ºC. Lineweaver-Burke Plots were plotted and the K_m and V_{max} of the immobilized enzyme were obtained from the slope and intercept of the graph.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 GOD immobilization in cross-linked PVA matrix : different casting temperature

For this part of the research, the immobilization method investigated was immobilization of glucose oxidase in cross-linked PVA matrix. Two different casting temperatures were studied, which were $4^{o}C$ and $25^{o}C$ to investigate the effects of casting temperature on the effectiveness of the enzyme immobilization procedure.

4.1.1 Ability of the membranes to retain glucose oxidase

In order to investigate the effectiveness of the immobilization method, which in this case was entrapment by cross-linked PVA at 0.06 cross-linking ratio and two different casting temperatures namely $4^{o}C$ and $25^{o}C$, protein contents of the washing solutions were determined. The washing solution (phosphate buffer) was changed

every 6 hours for the first 24 hours and every 12 hours thereafter. Biuret method was used to determine the total protein. The protein concentration determined from the experiment was used as an indicator for enzyme leakage, which led to the indication of the ability of the PVA-GOD membrane in retaining the glucose oxidase. Figure 4.1 shows that the total protein concentrations of the washing solutions for the PVA-GOD membranes demonstrate a declining profile for the whole period of investigation both for membranes immobilized at temperature 25°C and 4°C. Within 36 hours, the protein concentrations have reached zero. No sign of protein, and hence glucose oxidase, was in the washing solution. This indicates that the immobilization method was effective enough in immobilizing glucose oxidase in cross-linked PVA membranes.

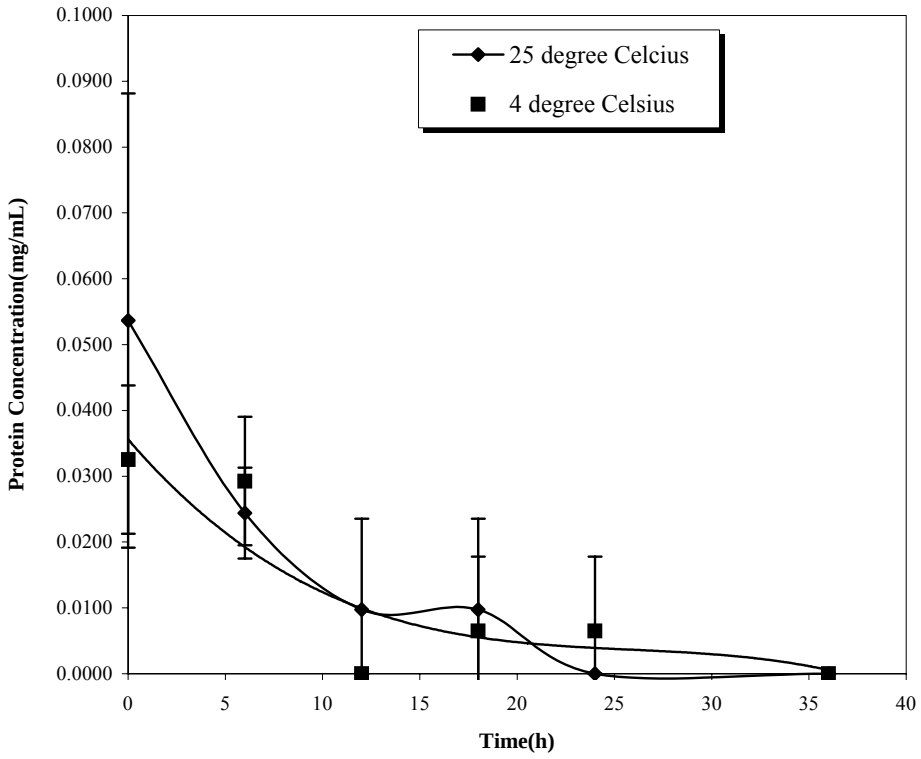

Figure 4.1 Leaking profiles for PVA-GOD membranes immobilized at 25°C and 4°C.

As a rough comparison between the PVA-GOD membranes immobilized at 25°C and 4°C, the enzyme leakage of the membranes immobilized at 25°C was higher during the initial investigation period compared to membranes immobilized at 4°C. However, the membranes immobilized at 25°C stopped leaking earlier than the membrane immobilized at 4°C.

4.1.2 Stability of the membranes

The apparent activity of the immobilized glucose oxidase immobilized in cross-linked PVA was determined using colourimetrical enzyme assay based on the oxidation of o-dianisidine through a peroxidase-coupled system. The dye produced from the reaction resulted in the colour intensity of the assay solution, which was determined photometrically at 450nm [65]. Furthermore, the stability of the repeated-use PVA-GOD membranes was examined as well as the decay of apparent enzyme activity over time and limited lifetime of the enzyme layer of a biosensor have been reported [66]. The enzyme activity of the membranes was tested within 25 storage days. The membranes were prepared and stored in phosphate buffer at 4 °C. The first enzyme assay was carried out on membranes that had been stored for 3 days.

Figure 4.2 shows the apparent enzyme activity of the membrane immobilized at 25°C and 4^{o}C. From the graph, it can be observed that for both types of membranes, the activity of the enzyme increased from day 3 to about day 10 before becoming rather stable from day 10 to day 25. This increase might be due to diffusion problems as the membranes, which are hydrogels, might not still be at their equilibrium states in terms of water content. The diffusion property of a membrane can be examined by determining the water content of the PVA-GOD membrane. From the water content, the mesh size or the cross-linking density can be estimated.

A high water content membrane indicates low cross-linking density and thus high permeability of the membrane.

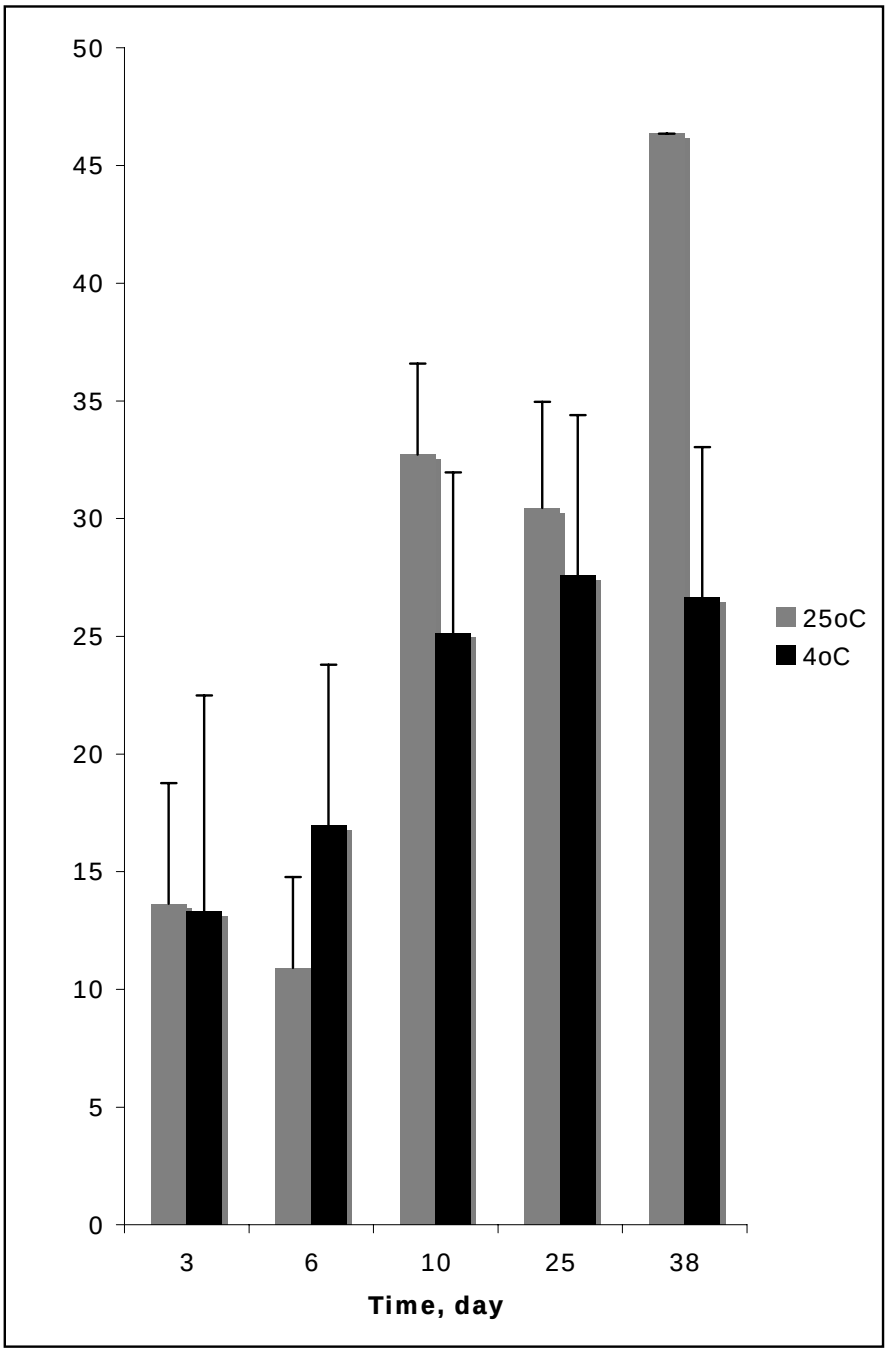

Figure 4.2 Comparison of apparent enzyme activity for PVA-GOD membranes immobilized at 25°C and 4°C.

The reason the membranes were immobilized at lower temperature was to reduce the exposure of the enzyme to high temperature in order to preserve the enzyme activity. Longer exposure of enzyme to high temperature will denature the enzyme and reduce the enzyme lifetime. From day 10 to day 25, it was observed that the enzyme activity of the membrane at temperature 25°C was higher than the membrane immobilized at 4°C, although the opposite was expected. This might due to insufficient time for the polymerization process at the lower temperature even though the membranes formed at lower temperature were allowed to polymerize for an additional 12 hours compared to the membranes immobilized at 25°C. The different polymerization procedures might have resulted in membranes with different water contents thus the membranes might have different cross-linking ratios and thus different properties.

4.2 GOD immobilization in cross-linked PVA matrix: different PVA concentration

For this part of the research, the immobilization method investigated was immobilization of glucose oxidase in cross-linked PVA matrix. Two different concentrations of PVA we were studied, which were 10% PVA and 15% PVA to investigate the effects of matrix concentrations on the effectiveness of the enzyme immobilization procedure.

4.2.1 Ability of the membranes to retain glucose oxidase

For both types of membrane, 10% and 15% PVA matrix, the cross-linking ratios were 0.06. As indicated earlier, after an enzyme immobilization process, the enzymes that were not properly immobilized will leak out from the membrane. Usually, immobilization process that employs chemical cross-linking agents will result in membranes with comparatively short leaking period. Figure 4.3 shows that leaking profile for GOD immobilized in 10% and 15% PVA at the cross-linking ratio of 0.06. The period of leaking was shorter if higher concentration of PVA (15%) was used. This suggests that with higher concentration of PVA, more matrices were available to entrap the enzyme, thus suppressing the leaking more effectively.

The total amount of the equivalent protein leaked from enzymatic membranes prepared using 10% PVA was 20% higher than the amount leaked from enzymatic membranes prepared using 15% PVA. Its period of leaking was also 18 hours longer respectively.

4.2.2 Stability of the membranes

Figure 4.4 shows that the apparent activities of the immobilized enzyme became stable at around day 10. As explained earlier in 4.2 the increase in activity from day 3 to day 10 might be due to hydration equilibrium problems. Enzymatic membranes prepared using 10% PVA showed approximately 33% higher activity than the ones prepared using 15% PVA. This might be because even though the 15% PVA matrix was more effective in retaining the enzyme, the tighter matrix may impose greater diffusional barrier towards glucose and hydrogen peroxide diffusion and thus limiting the enzymatic membrane response.

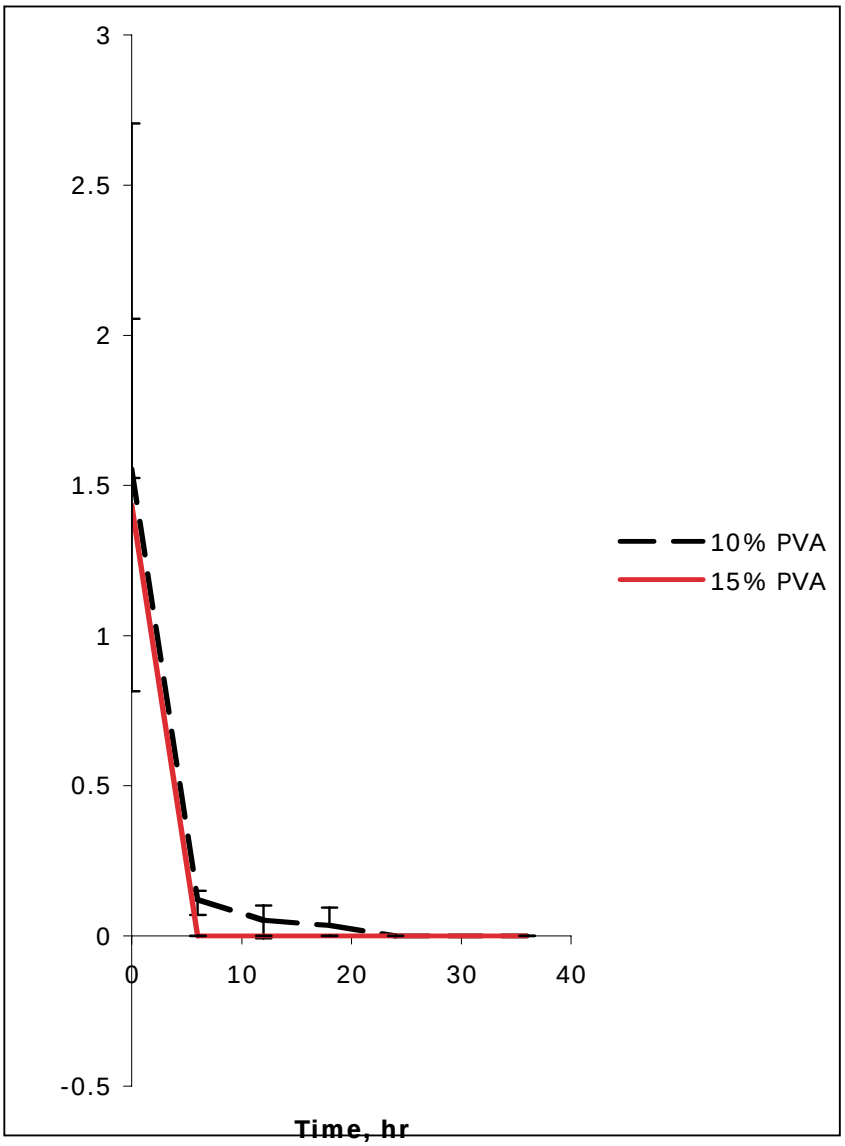

Figure 4.3 Leaking profiles PVA-GOD membranes immobilized with 10% and 15% PVA.

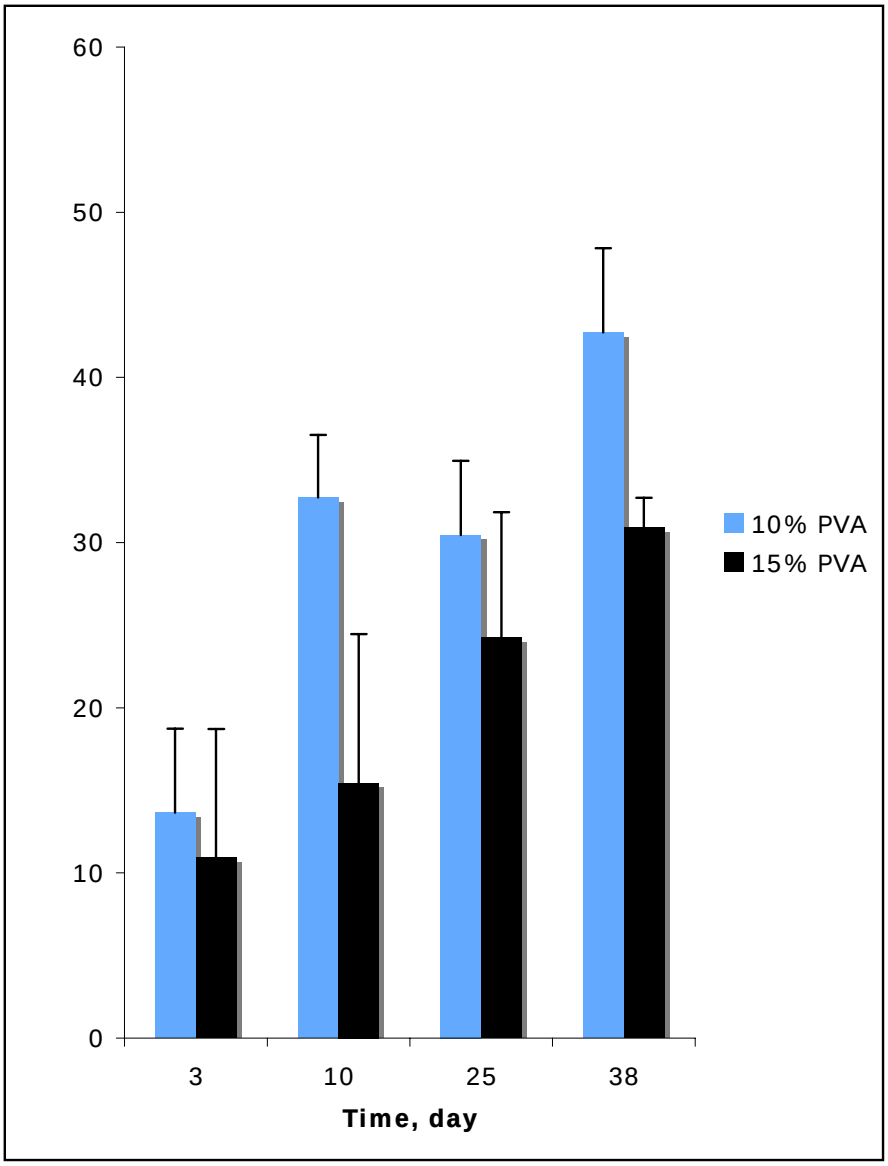

Figure 4.4 Stability of PVA-GOD membranes immobilized with 10% and 15% of PVA.

4.3 GOD immobilization in freeze-thawed PVA matrix

For this part of the research, the immobilization method investigated was immobilization of glucose oxidase in freeze-thawed PVA matrix. Compared to the two previous methods, this procedure is a physical method. Three different matrix concentrations were studied, which were 5% PVA, 10% PVA and 15% PVA to investigate the effects of matrix concentrations on the effectiveness of the enzyme immobilization procedure.

4.3.1 Ability of the membranes to retain glucose oxidase

BCA method, which is more sensitive than Lowry method, was used to determine the total protein contents in the washing solution. The total protein contents represent the amount of enzyme that leaked out of the membrane and thus become an indicator of the ability of the membranes to retain glucose oxidase. Figure 4.5 shows the leaking profile for freeze-thawed PVA-GOD membranes prepared with 5% PVA, 10% PVA and 15% PVA. PVA-GOD freeze-thawed membranes prepared with 5% PVA, 10% PVA and 15% PVA stopped leaking after 72 hours, 48 hours and 36 hours respectively. This indicates that the more concentrated the PVA used for the matrix, the higher the cross-links that were formed and the higher the amount of enzyme that was retained in the matrix.

4.3.2 Water contents of the membranes

One method that can be used to estimate the cross-link density of a cross-linked membrane is by measuring its water content. Figure 4.6 shows the relationship between water content and PVA concentrations of the PVA-GOD freeze-thawed membranes. As expected membranes prepared using 5% PVA have higher water contents than membranes prepared using 10% and 15% PVA. This suggest that membranes made with 15% PVA had the highest cross-link density followed by membranes prepared with 10% PVA and 5% PVA respectively. Water content is also a good indicator of the permeability of the membranes. The higher the water content, the lower the permeability of the membranes.

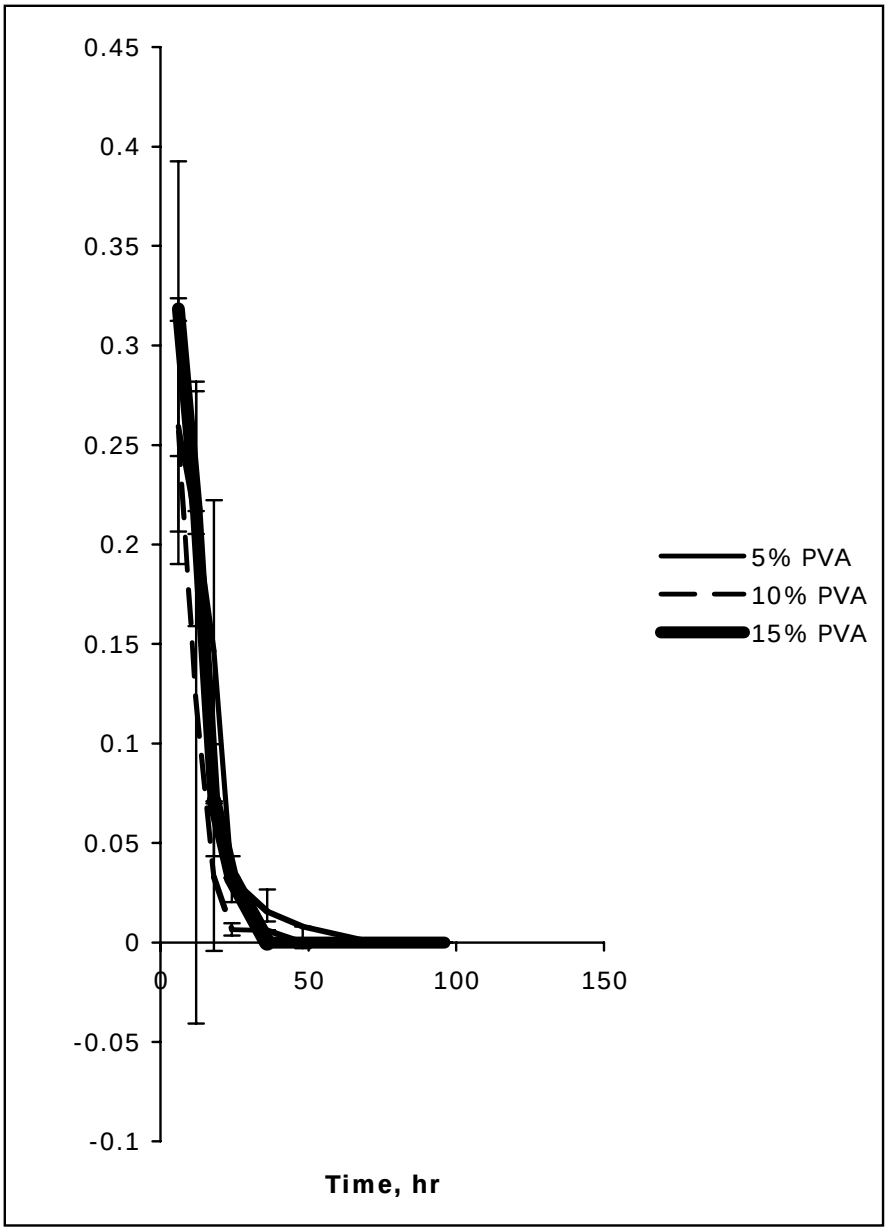

Figure 4.5 Leaking profiles for PVA-GOD freeze-thawed membranes prepared using 5% PVA, 10% PVA and 15% PVA.

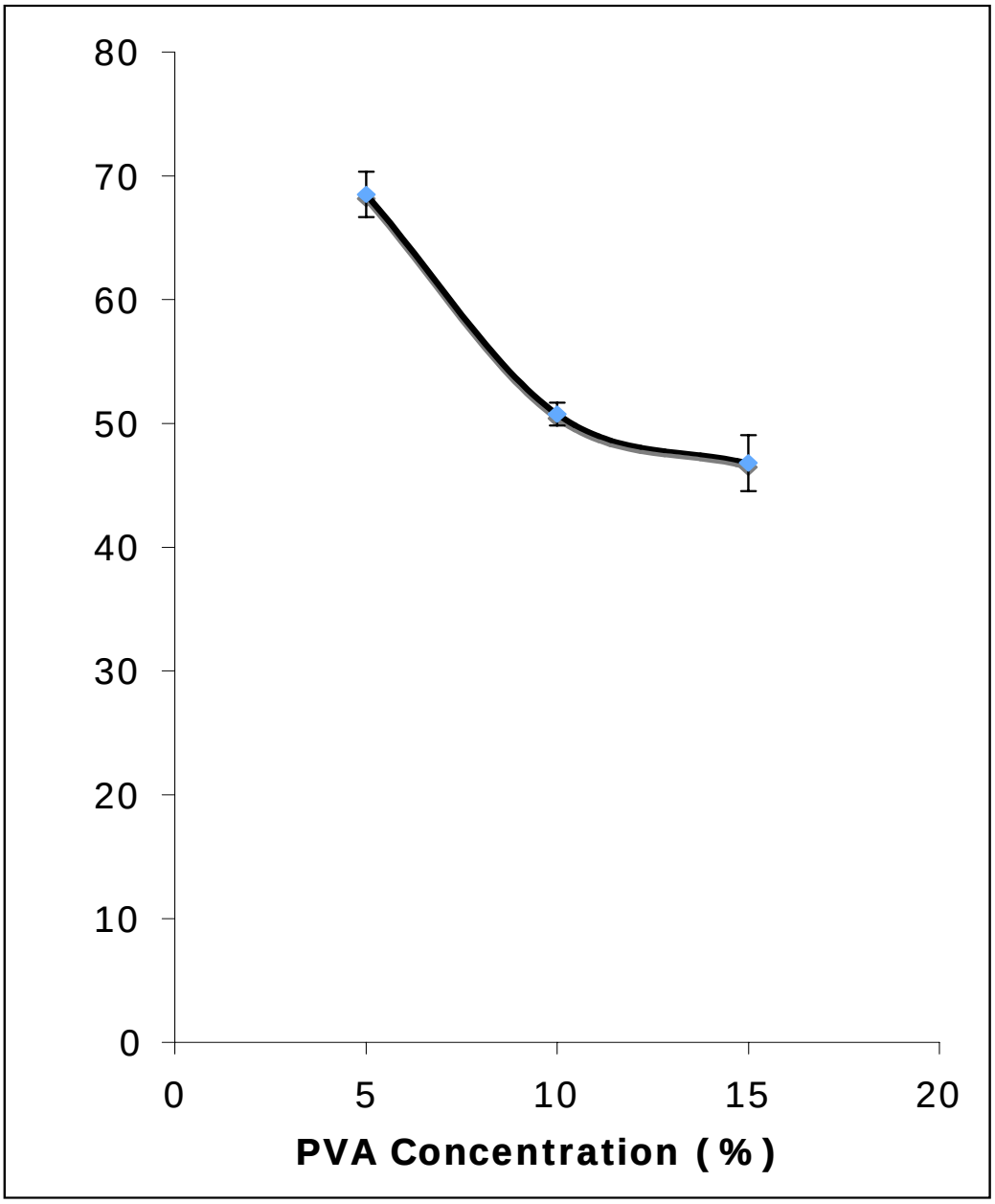

Figure 4.6 The relationship between the concentration of the PVA matrix of the freeze-thawed PVA-GOD membranes and their water contents.

4.3.3 Stability of the membranes

Apparent enzyme activities of the immobilized enzyme were detected using colorimetric method with o-dianisidine as the dye. The influence of storage in buffer solution and the effect of repeated-use on the apparent activities of the enzymatic membranes were studied. The first enzyme assays were tested after 4 days. Figure 4.7 shows the stabilities of the 5%, 10% and 15% freeze-thawed PVA-GOD membranes over time. Unlike the previous methods where chemical cross-linker was applied to cross-link the PVA, there was no increase in enzyme activity before the activity stabilized and finally decayed. Instead, the apparent enzyme activities of the membranes decayed over time as should be expected [66]. This might be due to the milder cross-linking conditions of the physical method, which resulted in higher water contents of the membranes and thus making it easier to reach equilibrium conditions.

10% freeze-thawed PVA-GOD membranes have the highest activities compared to the other two. This might be due to high cross-link density and moderate water content that resulted in membranes that can effectively entrapped the GOD while maintaining adequate permeability thus ensuring the appropriate microenvironment for the enzymes. The performances of 15% freeze-thawed PVA-GOD membranes and 5% freeze-thawed PVA-GOD was comparable. Even though 15% freeze-thawed PVA-GOD membranes suppressed enzyme leakage earlier than the others thus suggesting better enzyme retention; however its low water content indicated very high cross-link densities that might have adverse effects on membrane permeability and the conformational configuration of the immobilized enzymes. As expected, the 5% freeze-thawed PVA-GOD membranes were not very effective in retaining the enzymes due to low cross-link density but its high water content promote a good microenvironment for the immobilized enzymes.

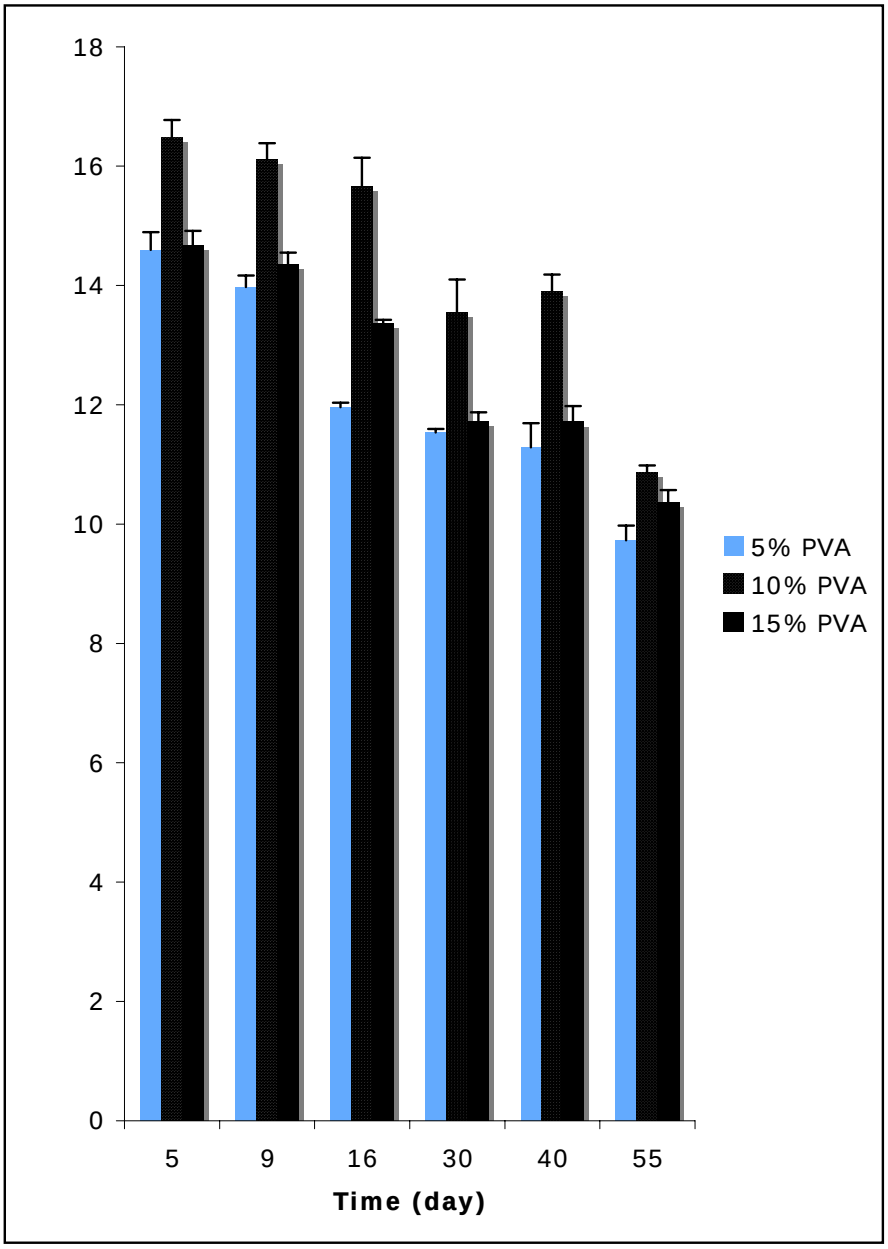

Figure 4.7 Stability of freeze-thawed PVA-GOD membranes prepared using 5% PVA, 10% PVA and 15% PVA.

4.3.4 Enzymatic membranes apparent kinetics

The apparent kinetic properties of the enzymatic membranes were obtained using Lineweaver-Burke plot. Apparent V_{max} for PVA-GOD membranes with 5%, 10% and 15% PVA was 13.3 mM/min, 26.12 mM/min and 17.53 mM/min respectively. The apparent Michaelis constant (K_m^{app}) for PVA-GOD membranes with 5%, 10% and 15% PVA was 4.8 mM, 5.95 mM and 5.15 mM, respectively. The low values indicated that the enzymes had a high affinity for substrate, as was usually the case for enzyme in solutions. This implied that the freeze-thaw physical immobilization method was a mild method that resulted in a good microenvironment for the enzymes. However, low K_m^{app} limits the detection limit of the biosensor that uses this type of enzymatic membrane as its biological recognition element. Other methods such as the use of diffusional outer membrane have to be employed to extend the linear range of substrate detection.

4.4 GOD immobilization in PVA/TMOS sol gel matrix

For this part of the research, the immobilization method investigated was immobilization of glucose oxidase in PVA/TMOS sol gel matrix. To improve the retention of enzyme in the matrix, (3-glycidoxypropyldimethylethoxy) silane (3GPDES), a cross-linker was used to cross-link the TMOS based sol-gel matrix. The work focused on the effect of cross-linker concentration on the performance of the enzymatic membranes.

4.4.1 Ability of the membranes to retain glucose oxidase

The washing solutions were analyzed for the presence of protein to investigate the effectiveness of immobilizing GOD in cross-linked silica sol and poly vinyl alcohol membrane incorporated with (3-glycidoxypropyldimethylethoxy) silane. Phosphate buffer solution was changed every 6 hours for the first day, every 12 hours for the second day and 24 hours thereafter and was analyzed for any sign of protein leakage. Using the Biuret method for determination of total protein, the leaking of glucose oxidase (GOD) in the washing solution was determined.

Figure 4.8 shows the total protein concentrations of the washing solution for the PVA/TMOS-GOD membranes for membranes with 1:1, 1:2 and 1:3 TMOS to 3GPDES volumetric ratios, respectively. Within 72 hours, the protein concentrations in the washing solutions for all three types of membranes have reached zero. This means that the immobilization method was effective enough to retain GOD in the cross-linked silica sol and PVA membrane incorporated with (3-glycidoxypropyldimethylethoxy) silane. The enzymatic membranes with the higher concentration of the cross linking agent [(3-glycidoxypropyldimethylethoxy) silane] was expected to show better enzyme retention. However, figure 4.4.1 shows that the leakage profiles for the three membranes were not much different indicating that the method was effective for all membranes.

4.4.2 Stability of the membranes

The immobilized membranes were placed in the phosphate buffer solution (pH 7) when not in used. The stability of the membranes was investigated using amperometric measurement where the glucose stock solution was injected into the

cell of a potentiostat with the membrane attached to the surface of the working electrode (platinum).

To determine the stability of the PVA/TMOS-GOD membrane, current response of the membranes to 5 mM glucose over time was recorded. Day 17 in Figure 4.9 signifies the seventeenth day after the membranes were stripped off from the glass slides. The first sixteenth day was to make sure that the membranes had reached stability.

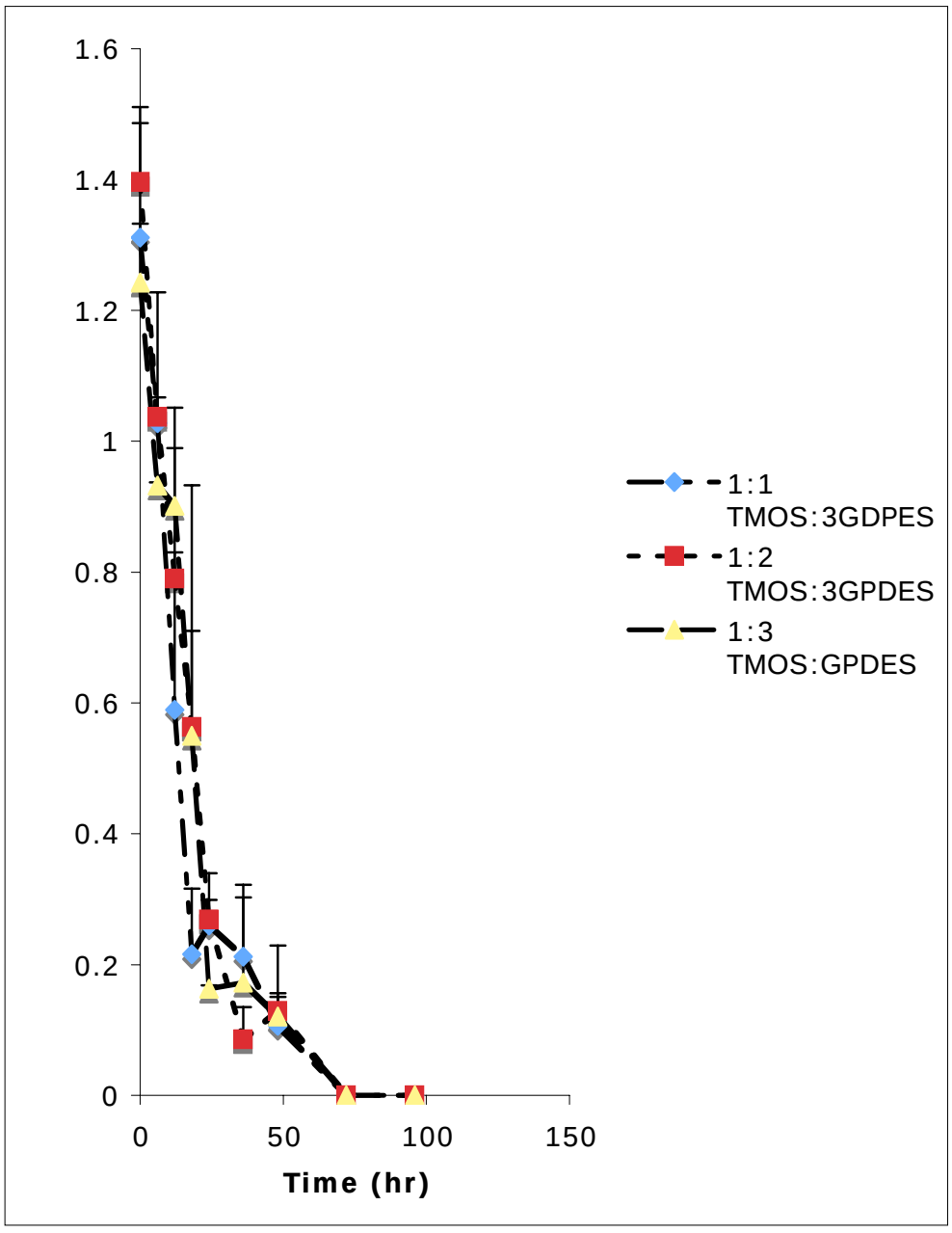

Figure 4.8 Leaking profile for PVA/TMOS-GOD membranes

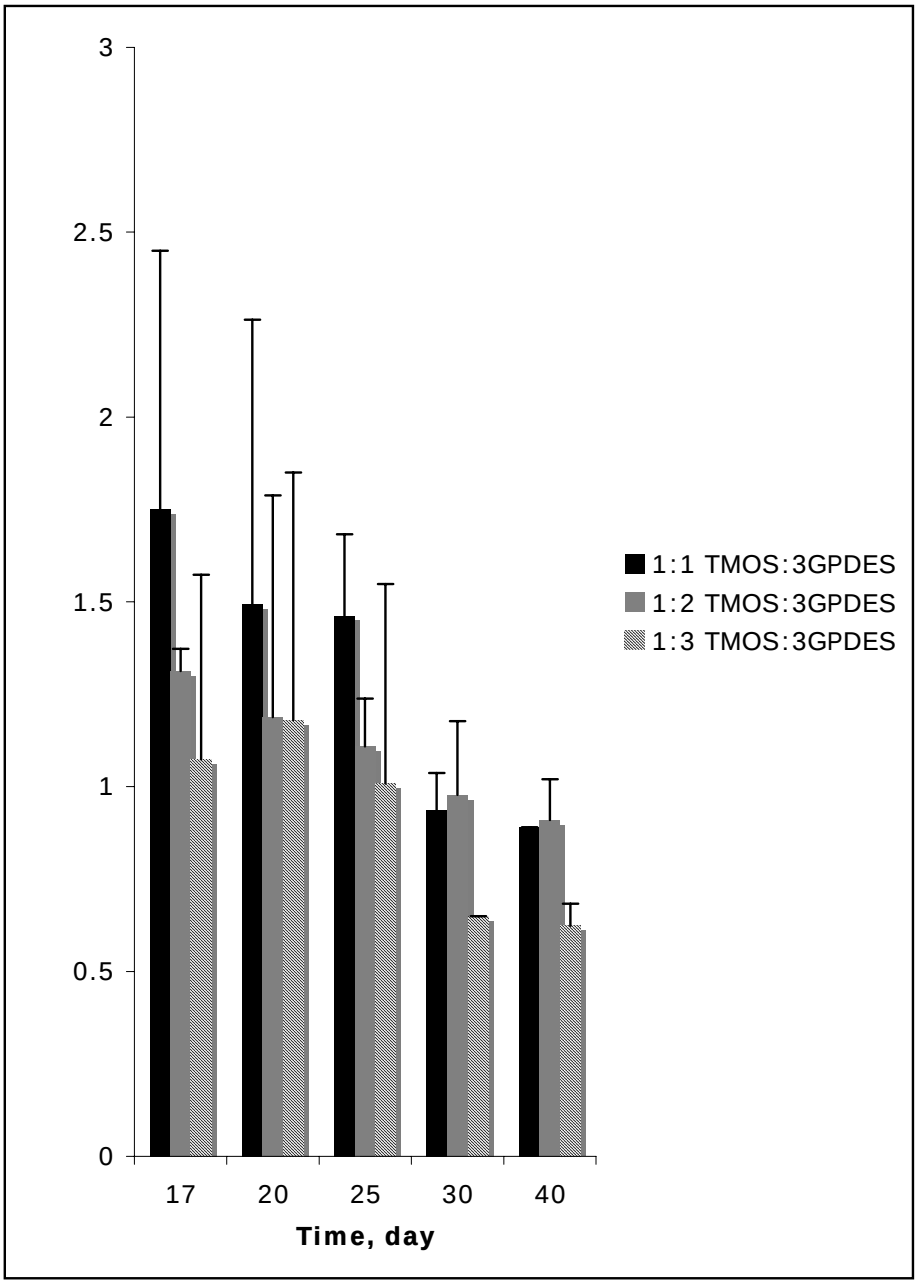

Figure 4.9 Stability of the PVA/TMOS-GOD membrane PVA/TMOS-GOD membranes.

Figure 4.9 shows that the activities of all three types of membranes decreased with time. As explained earlier, this decrease was due to denaturation of enzyme, which is common for all biosensors based on enzyme. Until day 25, membranes with 1:1 TMOS:3GPDES showed higher activities than the other membranes. Then

the activities decreased rather rapidly. At day 40 only 51% of the initial activity remained. Membranes with 1:2 TMOS:3GPDES were quite stable with 69% of the initial activity remained at day 40. For membranes with 1:3 TMOS:3GPDES, 58% of the initial activity remained at day 40.

4.4.3 Enzymatic membranes apparent kinetics

The apparent kinetics of the immobilized membranes was investigated using amperometric measurements. The kinetic parameters for the catalytic reaction, K_m and V_{max} were estimated from modified Lineweaver-Burke plot. The K_m and V_{max} of the enzyme immobilized in the membrane were obtained from the slope and intercept of the graph.

$$\text{Intercept} = \frac{1}{V_{\max}} \qquad \text{Slope} = \frac{K_m}{V_{\max}}$$

Both parameters are substrate specificity factors used to indicate catalytic efficiency and biosensor performance. High values indicate better biosensor performance.

At high glucose concentrations a platform response is observed, showing a characteristic of the Michelis-Menten kinetic mechanism. For 1:1 TMOS:3GPDES enzymatic membrane, the V_{max} value was 5.6 μA and the K_m value was approximately 1.5 mM. For 1:2 TMOS:3GPDES enzymatic membrane, V_{max} was 6.0 μA and K_m was approximately 1.8 mM. For 1:3 TMOS:3GPDES enzymatic membrane 3, the V_{max} and K_m values were 5.2 μA and 1.5 mM, respectively.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

5.1.1 GOD immobilization in cross-linked PVA matrix: different casting temperature

In this work, the basic working principle of a glucose biosensor especially the enzyme layer was studied. GOD was immobilized in cross-linked PVA matrix. The effect of casting temperature on enzymatic membrane performance was investigated.

The immobilization of glucose oxidase in cross-linked poly(vinyl alcohol) was shown to able to produce membranes with sufficient enzyme activity. The enzyme activity range detected by membrane immobilized at 4°C was 13.33mU/mL - 26.67mU/mL. The enzyme activity range for membranes immobilized at 25°C was 10.91mU/mL – 30.45mU/mL. The membranes immobilized at 25°C show higher enzyme activity in 25 days compared to membranes immobilized at 4°C. The membranes immobilized at 25°C were found to be able to retain enzyme better.

5.1.2 GOD immobilization in cross-linked PVA matrix: different PVA concentration

In this work, the effects of PVA concentration on the performance of cross-linked PVA-GOD membranes in terms of enzyme leakage and available activity were investigated. The cross-linker used was glutaraldehyde and the cross-linking ratio was 0.06. The experiments were conducted at room temperature.

With higher PVA concentration (15%), the enzyme retaining ability was better. For membranes prepared with 15% PVA, total enzyme leakage was 20% lower than the ones prepared with 10% PVA. The 10% PVA-GOD membranes leaked for 16 hours longer than the 15% PVA-GOD membranes. However, the 10% PVA-GOD membranes performed better in terms of available enzyme activity. Enzyme activity of the 10% PVA-GOD membranes was approximately 33% higher than the 15% PVA-GOD membranes. This can be attributed to better permeability and micro-environment of the 10% PVA-GOD membranes.

5.1.3 GOD immobilization in freeze-thawed PVA matrix

In this work, GOD was physically immobilized in PVA through the freeze-thaw method. The immobilization method was milder than using chemical cross-linker. The effect of PVA concentration on enzymatic membranes performance was investigated.

This work suggests that the higher the PVA concentration used for immobilization, the better the retention of the enzyme. Nevertheless, higher PVA concentration of immobilization didn't necessarily correlate well with enzyme activity. 10% freeze-thawed PVA-GOD membranes have the highest activities. This might be due to high cross-link density and moderate water content that resulted in membranes that can

effectively entrapped the GOD while maintaining adequate permeability thus ensuring the appropriate microenvironment for the enzymes.

The performances of 15% freeze-thawed PVA-GOD membranes and 5% freeze-thawed PVA-GOD was comparable. Even though 15% freeze-thawed PVA-GOD membranes suppressed enzyme leakage earlier than the others thus suggesting better enzyme retention; however its low water content indicated very high cross-link densities that might have adverse effects on membrane permeability and the conformational configuration of the immobilized enzymes. As expected, the 5% freeze-thawed PVA-GOD membranes were not very effective in retaining the enzymes due to low cross-link density but its high water content promote a good microenvironment for the immobilized enzymes.

In terms of kinetic properties, PVA-GOD freeze –thawed membranes prepared with 10% PVA exhibited the highest K_m^{app} compared to the others. This means that they are more suitable to be used as bio-recognition elements for glucose biosensors than the other two.

5.1.4 GOD immobilization in PVA/TMOS sol gel matrix

In this work, GOD was immobilized in PVA/TMOS sol gel matrix. A chemical cross-linker was used to improve retention of enzyme. The effect of cross-linker concentration on enzymatic membranes performance was investigated.

The immobilization of glucose oxidase in silica sol/poly vinyl alcohol membrane incorporated with (3-glycidoxypropyldimethylethoxy) silane was shown to be able to produce membranes which acceptable stability. For the membranes

prepared with 1:1 TMOS:3GPDES, the percentage of enzyme activity which remained at day 40 was about 51%. Meanwhile, for the membrane prepared with 1:2 TMOS:3GPDES and 1:3 TMOS:3GPDES, the percentage of enzyme activity which remained at day 40 was 69% and 58%, respectively.

From the kinetics studies, it was calculated that V_{max}^{app} and K_m^{app} values for membranes prepared with 1:1 TMOS:3GPDES were 5.7 μA and 1.5 mM, respectively. Meanwhile, V_{max}^{app} and K_m^{app} values for membranes prepared with 1:2 TMOS:3GPDES were 6.0 μA and 1.8 mM, respectively. For membranes prepared with 1:3 TMOS:3GPDES, the V_{max}^{app} and K_m^{app} values were approximately 5.2 μA and 1.5 mM, respectively. Thus membranes prepared with 1:2 TMOS:3GPDES is most suitable to be used as the bio-recognition element for a glucose biosensor due to its stability and kinetic properties.

5.2 Recommendations

Throughout the experiment, controlling the thickness and size of the membranes are rather challenging. However, producing membranes with uniform size and thickness are essential, as it will affect the available enzyme activity and permeability of the membrane.

In this work, the enzyme activity, kinetic and stability studies were investigated. But, in order to get a more comprehensive study of the immobilized glucose oxidase, it should include several other tests such as interference test, reproducibility test and optimization of the biosensor.

For the interference test, it should be done to detect possible effects of several common chemicals such as ascorbic acid and uric acid to the biosensor. This test is particularly important for enzymatic membranes meant for amperometric glucose biosensor.

Reproducibility test should be conducted to determine if the enzymatic membranes are able to respond to the varying glucose concentration in an acceptable time and with reproducible output response.

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